From a non-targeted metabolomics approach to a targeted biomarkers strategy to highlight testosterone abuse in equine. Illustration of a methodological transfer between platforms and laboratories

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From a non-targeted metabolomics approach to a targeted biomarkers strategy to highlight testosterone abuse in equine. Illustration of a methodological transfer between platforms and laboratories

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Abstract

In order to overcome the challenge associated with the screening of Anabolic-Androgenic Steroids abuses in animal competitions, a non-targeted liquid chromatography coupled to high resolution mass spectrometry based metabolomics approach was implemented on equine urine samples to highlight potential biomarkers associated with the administration of such compounds, using testosterone esters as model steroids. A statistical model relying on four potential biomarkers intensity could be defined to predict the status of the samples. With a routine application perspective, the monitoring of the highlighted potential biomarkers was first transferred into high-throughput liquid chromatography-selected reaction monitoring (LC-SRM). The model's performances and robustness of the approach were preserved and providing a first demonstration of metabolomics-based biomarkers integration within a targeted workflow using common benchtop MS instrumentation. In addition, with a view to the widespread implementation of such biomarker-based tools, we have transferred the method to a second laboratory with similar instrumentation. This proof of concept allows the development and application of biomarker-based strategies to meet current doping control needs.

KEYWORDS
AAS, doping, LC-HRMS, LC-SRM, metabolomics

1 | INTRODUCTION

Growth promoters are notoriously known to improve performances in animal competitions, especially for horses. Doping practices mainly aim at increasing strengths, recovery and respiratory capacity. Among the various families of chemicals potentially employed in that context, Anabolic-Androgenic Steroids (AAS) remain the predominant class of misused forbidden compounds.[1,2] These practices are associated with both ethical issues in sport and animal welfare concerns. Thus, the use of AAS as performance enhancers is strictly banned by the International Federation of Horseracing Authorities (IFHA) and the Fédération Équestre Internationale to protect animals' health and horse racing/sport integrity.[3] In this context, laboratories bring constant effort to improve performances of their analytical methods. Currently, direct detection of the active compounds or their respective metabolites is the strategy mostly applied to screen for potential misuse.

Doping approaches are constantly evolving, with the use of low dose cocktails,[4] endogenous steroids,[5] pro-hormones,[6] designer drugs,[7] or even gene doping.[8] Although present screening strategies are accurate and sensitive, the detection ability of such approaches remains challenging, especially when those compounds are unknown or exhibit short half-lives in the organism, thus reducing detection windows. Considering these limitations, new strategies using transcriptomics,[9,10] proteomics,[11,12] or metabolomics[13–15] approaches have been reported over the last past 15 years.[16] Based on the measurement of an effect rather than the compound itself or its direct metabolites, these approaches investigate physiological alteration of a biological system upon prohibited substances administration in order to detect specific potential biomarkers. Metabolomics, in particular, consists of the large-scale and high-throughput measurement in biological matrices of low molecular-weight metabolites (< 1500 Da). Investigating the corresponding metabolome was notably enabled by both improved liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) workflows and by large data processing and analysis tools.[17] Proofs of concept of the relevance of such approaches in an anti-doping context are available.[18–20] Despite the great breakthrough of metabolomics strategies and the *tremendous momentum* of the scientific community, some aspects of these approaches still need to be further developed in relation to data mining and processing or potential biomarkers identification.[21] Particularly, to anticipate the implementation of these approaches, it is important to address aspects related to method robustness and then transferability. To address the needs of the laboratories, harmonised non-targeted protocols have been developed.[13,22,23]

According to a recent publication of Kaufmann,[24] strategies relying on HRMS are now widely adopted in residues analysis field. In parallel, cost, associated with technical requirements, remains the reasons why analysis on triple quadrupole mass spectrometers (QqQ) in selected reaction monitoring (SRM) has been the most widely used strategy for routine applications currently. With both types of instruments now coexisting in laboratories, it is important to develop methods that are quickly and easily transferable from one platform to another regardless within or between laboratories.

To date, this type of transfer has been little documented in the scientific literature. In particular, examples reporting the monitoring of biomarkers, initially discovered using an HRMS system in full scan mode, with an alternative LRMS platform in a targeted acquisition mode such as SRM remain scarce. The advantage of such monitoring is its robustness associated with its confidence given from the signal in terms of selectivity. This type of transfer is therefore expected by the community and constitutes a major bottleneck when structural identification of the revealed potential biomarkers remains not fully completed.

The present study was inspired of a previous work conducted by Dervilly et al.[13,22] on the application and the validation of a new tool based on metabolomics to screen for β-agonist misuses in calves. Following the same approach, the first aim was the application of state-of the art metabolomics strategy to reveal potential biomarkers signing the administration to a horse of a testosterone esters cocktail. A statistical model based on four potential biomarkers was established allowing classification of sample status. Then, a LC-HRMS method was further developed to monitor the four markers and compared to a direct testosterone screening. The robustness of the analytical strategy and the classification model performances were both evaluated whilst transferring from LC-HRMS to LC-LRMS instrumentation. Finally, the method was successfully transferred to a second laboratory to evaluate the inter-platform robustness. This proof-of-concept demonstrates the feasibility of the whole process from potential biomarkers selection to analytical method implementation in a doping control context.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Isotope labelled standards, namely, leucine-5,5,5 d3, L-tryptophan-2,3,3 d3, 3-indole-2,4,5,6,7 d5-acetic acid and 1,14-tetradecanedioic d24-acid were purchased from Steraloids (Newport, RI, USA) and Sigma-Aldrich (St. Louis, MO, USA). An internal standards (IS) mixture at 5 ng/μL each was prepared in a mixture of water/ethanol (25/75, v/v). Testosterone d3 and testosterone sulphate d3 were purchased from National Measurement Institute (NMI, Pymble, Australia). Reference compounds (testosterone and testosterone sulphate) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Steraloids (Newport, RI, USA). All compounds were prepared at a concentration of 1 mg/mL.

All solvents (LC-MS grade) used in this study (acetonitrile, ethanol, methanol, water, acetic acid and lithium chloride) were obtained from Sigma-Aldrich Chromasolv Reagents (St. Louis, MO, USA). Ammonium acetate anhydrous powder was purchased from Sigma-Aldrich Chromasolv Reagents (St. Louis, MO, USA). Two ammonium acetate solution (pH = 7.2) at concentration of 250 and 50 mM were prepared. SPE isolute C18 of 1 g sorbent was purchased from Biotage (Hengoed, UK).

MSCAL5 ProteoMass™ LTQ/FT-Hybrid ESI Pos/Neg (Sigma-Aldrich) (Calmix-positive, for the positive-ionisation mode, consisting of caffeine, L-methionyl-arginyl-phenylalanyl-alanine acetate and Ultramark 1621; Calmix-negative, for the negative-ionisation mode, consisting of the same mixture plus sodium dodecyl sulphate and sodium taurocholate) was used for the external calibration of the MS instruments.

2.2 | Biological samples

One 4 years old gelding weighing 496 kg received a single intramuscular injection (2 mL) of Sustanon, a testosterone esters cocktail solution (testosterone propionate (30 mg/mL), testosterone phenylpropionate (60 mg/mL), testosterone isocaproate (60 mg/mL) and testosterone decanoate (100 mg/mL)) (Oregon, lotsB-NO-0240 and B-NO-0267). Urine samples were collected over the 6 days

preceding the administration and regularly all along the animal phase until 216 days after the single administration, for a total of 49 time points ($t = 49$). All experimental procedures were completed in accordance with European guidelines for use and care of animals.[25]

Quality control (QC) sample was prepared by pooling equal volumes (500 µL) of the 47 urine samples. QC controls were dispatched along the analytical sequence every five samples according to metabolomics good practices.[26]

Control urine samples ($n = 30$) were randomly selected from negatively tested urine samples including biological variability regarding age, sex and practice. Their control status (i.e., compliant) was confirmed by appropriate analysis with ISO 17025 accredited methods.

Urine aliquots (1 mL) of each collected and QC samples were stored at −20°C until analysis.

2.3 | Non-targeted metabolomics study

2.3.1 | Sample preparation

Firstly, aliquots of urine specimens (1 mL) were centrifuged (13,000 g; 4°C; 5 min). As reported by common metabolomics protocols,[27–30] 500 µL of the supernatant were normalised through water dilution according to their specific gravity (SG) measured by refractometry (ATAGO PAL-10S Thermo Fisher scientific). The normalised urine was subsequently filtered through a 10 kDa molecular weight cut-off polyethersulfone membrane under centrifugation (13,000 g; 4°C; 30 min). Finally, 10 µL of IS at 5 ng/µL was dried under nitrogen flow and taken up in 30 µL of filtrated urine.

2.3.2 | LC-HRMS fingerprinting

A Dionex Ultimate TM 3000 ultra-high performance liquid chromatography (UHPLC) system (Dionex Softron®, Germering, Germany) was equipped with a Hypersil Gold C_{18} column (100 mm × 2.1 mm i.d., 1.9 µm particle size). The column ovens and the sample trays temperatures were set to 35°C and 4°C, respectively. Solvents used were water containing 0.1% acetic acid (v/v) (A) and acetonitrile containing 0.1% acetic acid (v/v) (B). The flow rate was 400 µL/min. The elution gradient was set as follows: 95/5 A/B for 2.40 min, 75/25 A/B at 4.50 min, 30/70 A/B at 11.00 min, 100% B at 14.00 min to 16.50 min and finally, 95/5 A/B at 19.00 min to 25.00 min (Figure 1a). The injection volume was set to 2 µL.

The UHPLC system was coupled to a Q-Exactive mass spectrometer (Thermo Scientific®, Bremen, Germany) equipped with a heated electrospray ionisation (HESI) source operating in positive and negative modes. The acquisition of the raw data was performed using a MS1 full scan mode within the *m/z* 65–1000 range at a resolving power of 70,000 at *m/z* 200. Data acquisition was settled with an automatic gain control (AGC target) of 5.10^5 and a C-Trap inject time of 20 ms. ESI ion source settings were set as follows: the spray

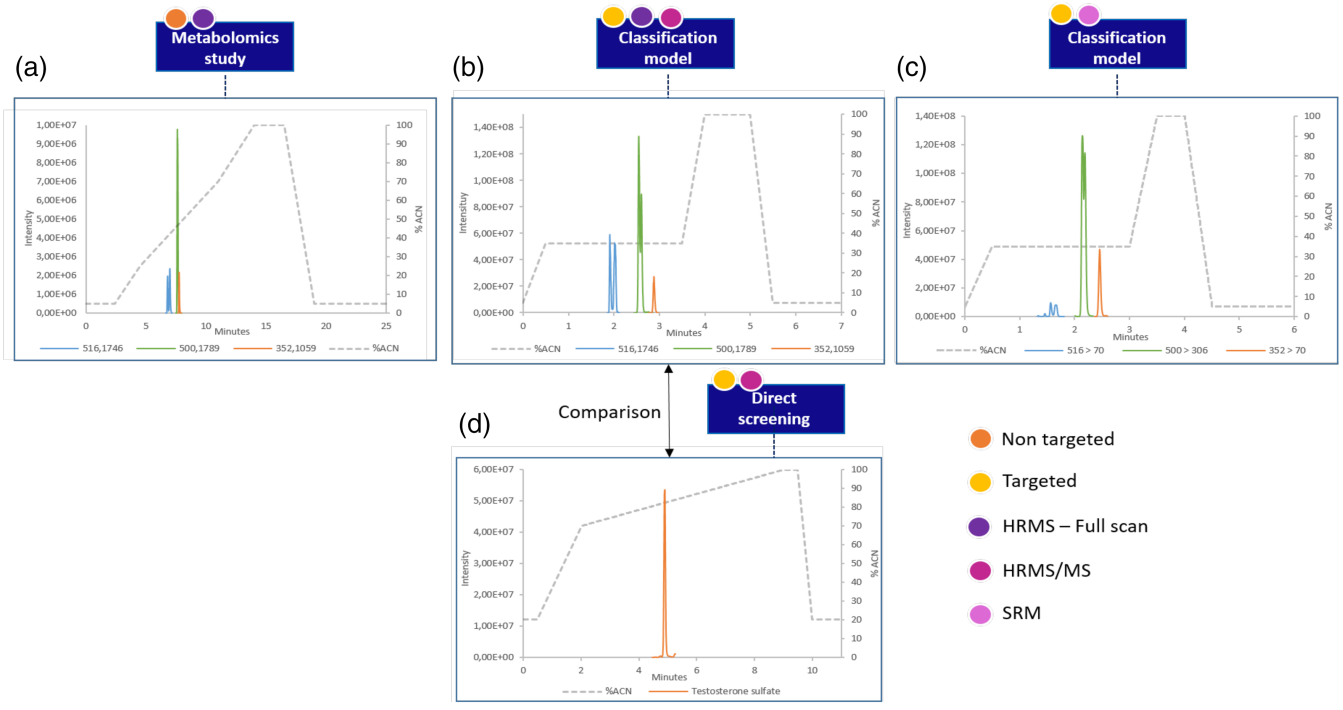


FIGURE 1 General workflow from the metabolomics study to a targeted potential biomarkers strategy to highlight testosterone esters abuse and comparison with a direct screening. Chromatographic gradients (a) used for the metabolomics study to highlight potential biomarkers, (b) used for the classification model in HRMS-full scan, (c) in SRM and (d) for the detection of testosterone sulfate in direct screening

voltage (+3 kV), the S-Lens RF level (50%), the tube lens voltage (+100 V), the capillary temperature (350°C), the heater temperature (200°C), the sheath gas pressure (50 arbitrary units), the auxiliary gas flow rate (20 arbitrary units) and the sweep gas flow rate (0 arbitrary unit). Full instrument calibration was performed using a MSCAL5 ProteoMass™ LTQ/FT-Hybrid ESI Pos/Neg.

2.3.3 | Data processing

Xcalibur V4.3 (Thermo Scientific®, Bremen, Germany) software was used for the generation of all chromatographic peaks acquired in full scan mode. Raw files were converted to mzXML format with MSConvert version 3.0.3347[31] and processed by XCMS package version 1.38.0 running under R version 3.0.2.[32] The MatchedFilter algorithm was used with the following parameters: step = 0.03, steps = 2, full width at half maximum (FWHM) = 15, signal/noise threshold (snthresh) = 3 and *m/z* difference (mzdiff) = 0.008. The "Obiwarp" method was selected for aligning peaks, and the "group density" function was used for grouping peaks following parameters: band width = 9, *m/z* width (mzwid) = 0.008, minimum sample necessary (minsamp) = 5 and maximum number of groups to identify in a single *m/z* slice (max) = 30. The resulting dataset table containing the extracted features assigned by their accurate mass to charge ratios, retention times and their relative abundances in each sample was then pre-filtered before data analysis as described previously. Briefly, all zero abundance values attributed to non-detected peaks were replaced for each feature with randomly generated values ranging between −30% and +30% of the lowest detected signal of the same selected feature.[33] A signal correction based on a locally estimated scatterplot smoothing normalisation was applied in data from QC analysis.[26] Then, annotation of features was performed with CAMERA,[34] and a filtration step was automatically performed aiming at eliminating the different ^{13}C isotopomers (M + 1, M + 2 and M + 3) and possible adducts of the same compound such as Na^+ and neutral losses. Finally, features abundances presenting a coefficient of variation (CV) > 30% in QC samples were discarded regarding their analytical irrelevance.

2.3.4 | Data analysis

Multivariate analysis was carried out using SIMCA software V13.0 (Umetrics, Umea, Sweden). A logarithmic transformation and Pareto scaling were applied before generating unsupervised principal component analysis (PCA) and supervised orthogonal projection of latent structures (OPLS) models. The PCA model aims at giving a general overview of the main discriminations observed and to investigate analytical drift based on QC clustering, whereas the OPLS model targets a supervised discrimination through the integration of a variable Y indicating the status of the observation. The validity and robustness of the models were evaluated by R2(Y) and Q2(Y) parameters, cross validation analysis of variance and finally, using permutation tests.

2.3.5 | Potential biomarkers structure investigation

To determine the nature of the ions observed, adducts of interest were studied by replacing the observed adducts with Li^+. This was achieved by mixing the LC outflow with 5 µL/min of an aqueous solution of lithium carbonate (5mM) using a tee. After a first filtration step described in 2.3.3, features were annotated by matching their accurate measured masses with theoretical masses. Mass tolerance of ±20 ppm was selected to shortlist more candidates from the following databases: Human Metabolome Database (https://hmdb.ca/), Bovine Metabolome Database (https://bovinedb.ca/), METLIN (Scripps Center for Metabolomics, https://metlin.scripps.edu), Drug Bank (Open Data Drug&Bank target Database, https://go.drugbank.com/), mzCloud (Advanced Mass Spectral Database, https://www.mzcloud.org), Mass Bank (High Quality Mass Spectral Database, https://massbank.eu/MassBank) and SIRIUS 4.8.2 (https://bio.informatik.uni-jena.de/software/sirius/).[35]

2.4 | Targeted monitoring

2.4.1 | From targeted LC-HRMS to LC-SRM analyses

LC-HRMS-Platform1 method optimisation and LC-PRM parameters

A Vanquish UHPLC system (Thermo Fisher Scientific®, Bremen, Germany) was used. Chromatographic separation was optimised based on the Guillarme et al approach.[36] According to the retention times of each molecule, their corresponding acetonitrile elution percentage was optimised. The elution gradient was finally set as follows: 95:5 A/B for 0.5 min, 65:35 A/B from 0.5 min to 3.5 min, 0:100 A/B at 4 min and finally, 95:5 A/B at 6 min to 7 min (Figure 1b). The injection volume was set to 5 µL.

LC systems were coupled to a Q-Exactive mass spectrometer (Thermo Scientific®, Bremen, Germany) equipped with a HESI source operating in positive mode. The spectrometric acquisition parameters implemented when running the Parallel Reaction Monitoring (PRM) method were the same as described above (section 2.3.2). Ions were selected within a 4 *m/z* quadrupole isolation window and fragmented in the Higher energy Collision-induced Dissociation (HCD) cell with a normalised collision energy (NCE) of 30%. AGC target was set to 2.10^5 and a C-trap inject time of 100 ms. MS/MS spectra were acquired at mass resolving power of 17,500 FWHM (at *m/z* 200). The potential biomarkers were characterised separately in full scan MS and PRM modes in order to study their fragmentation patterns for subsequent transitions selection in liquid chromatography-selected reaction monitoring (LC-SRM) acquisition.

LC-SRM parameters

An Acquity Ultra Performance Liquid Chromatography (UPLC®) System (Waters®, Milford, MA, USA) equipped with the same column, solvents (A and B) and flow rate as described above (section 2.3.2) for LC-HRMS method was used for chromatographic separation. The

elution gradient was adapted to the LC-SRM device chromatography chain as follows: 95/5 A/B at the beginning of the run, 65/35 A/B from 0.5 min to 3.0 min, 0/100 A/B from 3.5 to 4.0 min and finally, 95/5 A/B from 4.5 min to 6.0 min (Figure 1c). The injection volume was 5 μL.

The UPLC® system was coupled to a Xevo TQ-S triple quadrupole (QqQ) mass spectrometer (Waters®, Milford, MA, USA) equipped with an ESI source used in positive mode (ESI+). The acquisition transitions defined upon HRMS fragmentation study were optimised in the SRM mode. Cone voltage and collision energy were optimised individually for each putative biomarker (Table 1). Desolvation gas temperature was set at 600°C and flow rate at 1200 L/h, nebulising gas pressure at 7.0 bars, source housing temperature at 150°C and capillary voltage at +3.0 kV; argon was used as collision gas at 0.15 mL/min.

2.4.2 | Methods performances evaluation

Repeatability of the measurements was evaluated based on the detection of the metabolites contained in 14 QC samples and injected all along a batch as described earlier.[26,37] Peak area and retention time repeatability were evaluated considering the relative standard deviation obtained. Finally, the QC pools were serially diluted in water (1 QC, 0.5 QC, 0.25 QC, 0.125 QC, 0.1 QC and 0.05 QC) and subsequently analysed in three replicates. A dynamic range of detection was then established based on the normalised peak areas of the potential biomarkers in the QC and the serially diluted QC sample to address linearity and a putative ion suppression of the developed protocol for each potential biomarker.

2.4.3 | Method transfer from HRMS-Platform1 to HRMS-Platform2

A Dionex Ultimate TM 3000 UHPLC system (Dionex Softron®, Germering, Germany) (Laboratoire d'Etude des Résidus et Contaminants dans les Aliments) was used in the second platform (HRMS-Platform2). Same chromatographic separation and full MS parameters as described in the

LC-HRMS-Platform1 method optimisation and LC-PRM parameters section were applied in the second platform.

2.4.4 | Targeted data processing

LC-HRMS and LC-SRM raw data files were initially processed with Xcalibur V4.3 and MassLynx V4.3 (Waters®, Milford, MA, USA), respectively. Subsequently, they were processed using Skyline 20.2 software.[38] For LC-HRMS in full scan acquisitions, the parameters of the transitions setting were as follows: prediction of the precursor mass (monoisotopic), collision energy (none), filter ion types (p [precursor]), match tolerance *m/z* (0.005), precursor mass analyser: orbitrap with resolving power of 70,000 FWHM (at *m/z* 200) and include all matching scans. For LC-SRM acquisitions, the parameters of transitions setting were as follows: prediction of the precursor and product ion mass (average), filter ion types (p and f [precursor and fragment]) and method match tolerance *m/z* (0.5).

Dynamic range of detection was analysed based on linear regressions processed in R without applying normalisation method or regression weighting. For classification model development, preprocessing after peak integration consisted of a logarithmic transformation, pareto scale and a signal correction based on LOESS method as described in paragraph 3.3.

2.5 | Direct screening of testosterone

2.5.1 | Sample preparation

The sample preparation was based on a previously described method with minor modifications.[39] Urine aliquots (1 mL) were spiked with testosterone sulphate d3, used as an IS, at a concentration corresponding to 100 ng/mL of free testosterone and were subsequently diluted with 1 mL of 250mM ammonium acetate buffer and 2.5 mL of water. Samples were then loaded into C_{18} cartridges previously conditioned with 5 mL of methanol and equilibrated with 5 mL of ammonium acetate buffer (50 mM). Following a washing step consisting of a 5 mL mixture of ammonium acetate buffer (50mM)/methanol (60/40, v/v) and a desalting step with 5 mL of water/methanol (95/5, v/v), elution was performed using 5 mL of methanol. The eluate was then evaporated under gentle nitrogen stream at 45°C, transferred to LC vial and reconstituted in 50 μL of a mixture of methanol/water (80/20, v/v) prior to analysis on LC-HRMS.

TABLE 1 Retention times, SRM transitions monitored and collision energies of the four potential biomarkers using LC-SRM experiment

Biomarkers	Rt (min)	Cone (V)	Quantification transition	CE (eV)	Identification transition (Q1)	CE (eV)	Identification transition (Q2)	CE (eV)
M516a	1.91	25	516.2 > 340.1	25	516.2 > 70.0	20	516.2 > 498.2	15
M516b	2.01	25	516.2 > 340.1	25	516.2 > 70.0	20	516.2 > 498.2	15
M500	2.67	30	500.2 > 324.1	20	500.2 > 70.0	25	500.2 > 306.1	20
M352	2.79	25	352.1 > 219.1	25	352.1 > 70.0	25	352.1 > 139.1	25

2.5.2 | LC-PRM parameters

A Vanquish UHPLC system was equipped with a Raptor C_{18} column (150 mm × 3 mm i.d., 2.7 μm particle size). The column oven and the sample trays temperatures were set to 45°C and 4°C, respectively. Solvents used were water containing 0.1% formic acid (A) and methanol containing 0.1% formic acid (B). The flow rate was set at 600 μL/min. The elution gradient was as follows: 80:20 A/B for 0.5 min, 30:70 A/B at 2 min, 0:100 A/B at 9 min to 9.5 min and finally, 80:20 A/B at 10 min to 11.00 min (Figure 1d). The injection volume was set to 10 μL.

The UHPLC system was coupled to a Q-Exactive mass spectrometer equipped with a HESI source operating in negative mode. The acquisition of the raw data was performed using a PRM mode within the *m/z* 150–750 range at a resolving power of 35,000 at *m/z* 200. Data acquisition was settled with an automatic gain control of 2.10^5 and a C-Trap inject time of 100 ms. NCE was set at 65. The acquisition spectrometric parameters were as follows: the spray voltage (−0.5 kV), the S-Lens RF level (100), the tube lens voltage (+100 V), the capillary temperature (350°C), the heater temperature (425°C), the sheath gas pressure (40 arbitrary units), the auxiliary gas flow rate (10 arbitrary units) and the sweep gas flow rate (0 arbitrary units). Full instrument calibration was performed using a MSCAL5 ProteoMass™ LTQ/FT-Hybrid ESI Pos/Neg. Xcalibur V4.3 software was used for the generation of all chromatographic peaks acquired in PRM mode.

2.5.3 | Data processing

Data processing was performed on Skyline as described above. Then, peak area was normalised based on the spiked IS (testosterone sulphate area/testosterone sulphate d3 area). The testosterone concentration in the sulphated fraction was then estimated by semi-quantification based on the peak area measured for testosterone sulphate and the peak area obtained for the IS, associated with the free testosterone concentration initially added to the sample.

3 | RESULTS AND DISCUSSION

3.1 | Non-targeted metabolomics to highlight potential effect biomarkers of testosterone esters

3.1.1 | Data quality and OPLS model establishment

The quality of UHPLC-HRMS data is of major importance when performing a non-targeted investigation of the metabolome to provide accurate and unbiased interpretation of the analytical outcome.[40] As depicted in Figure S1, QC samples were successfully grouped, compared to the total variability observed, confirming the analytical robustness.[37,41] The sample collected at D145 was considered as an outlier in this study and was subsequently excluded (Figure S1). A careful review of IS signals confirmed the stability of the retention time, peak area and the mass accuracy which remained below 5 ppm. As negligible analytical drift was observed after data treatment, the whole dataset was valid for further statistical analyses.

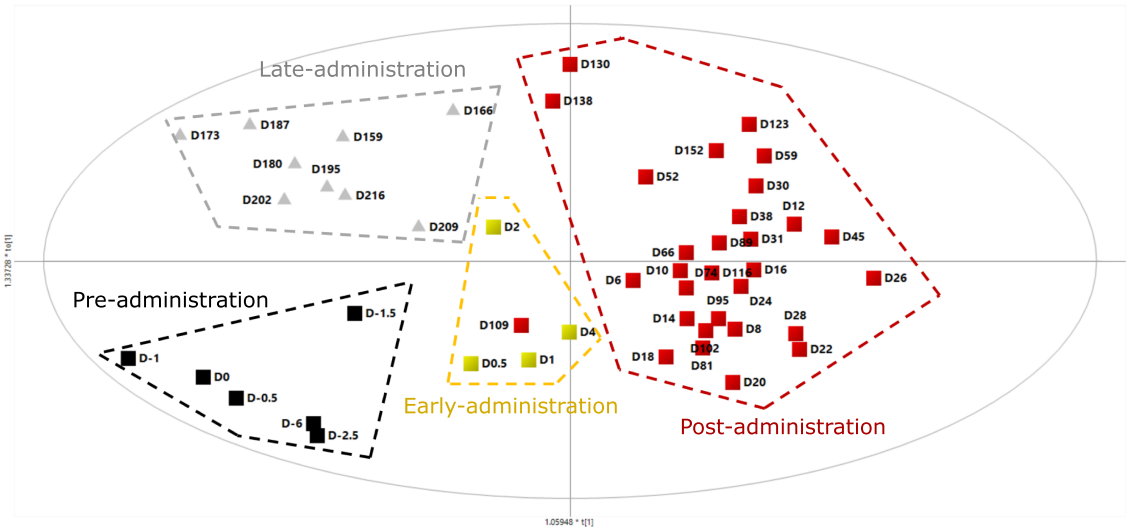
A single animal experiment involving an AAS administration was selected in the frame of this study to investigate global effects of testosterone esters administration on the horse metabolism for more than 200 days. Urine samples collected before and after testosterone esters administration ($t = 49$) were characterised. A final dataset table containing 2735 features was generated and subjected to multivariate statistical analysis.

Samples collected before the administration of testosterone esters were assigned to the control group ($t = 6$), whilst those collected after the administration constituted the treated group ($t = 43$). Modelling the status of both groups was performed using supervised OPLS, which allowed generating a descriptive model (Figure 2) exhibiting the following performances: R2(X) = 0.22, R2(Y) = 0.74 and Q2 = 0.40. It allowed evidencing four distinct clusters of samples, corresponding respectively to (i) pre-administration collected urine samples (D-6 to D0), (ii) very first days after the administration (D1 to D4), (iii) urine samples collected between 5 and 158 days following administration and (iv) urine samples collected at the end of the animal experiment (159–216 days). Samples collected within the 5–158 days post-administration range were specifically plotted on one side of the OPLS leading to an efficient separation from the rest of experiment samples and attesting for significant disrupted global urinary profile over 5 months following testosterone esters administration. Furthermore, urine samples collected after 159 days post-administration were plotted close to the pre-administration samples highlighting a return to the equilibrium state at the end of the experiment. A single post-administration sample, D109, was plotted in the group of early post-administration samples. A possible physiological or environmental impact on the horse metabolism could be a hypothesis for such misclassification.

The obtained model clearly illustrates the chronological changes of the urinary metabolome profile over the animal experiment duration. It highlights the potential of such an approach to evidence the abuse of testosterone esters for more than 5 months after single administration.

3.1.2 | Discovery of testosterone esters potential biomarkers and classification model development

To highlight the most predictive features out of the filtered dataset, an S-plot of the OPLS model was generated allowing the selection of 171 relevant features (Figure S2). Among this selection, the individual kinetic profiles were reconstructed. Four features were subsequently highlighted through critical assessment of their respective analytical profiles and abundances (a minimum of 10 kinetic points each, after administration, presenting a feature abundance fold change > 5 in comparison with pre-administration group feature abundance). Such strategy has been already used for potential biomarkers selection.[42] In addition, these features presented relevant contribution in the



R2(X)=0.22, R2(Y)=0.74 and Q2=0.40

FIGURE 2 Orthogonal projection to latent structures (OPLS) based on 2735 features. OPLS model was generated between control (samples before administration of testosterone esters) and treated group (samples after administration of testosterone esters). Analysis of urine samples from the *in vivo* study was performed on UHPLC (Ultimate TM 3000)-Q-Exactive (Thermo Fisher Scientific) in full scan mode and normalisation (LOESS), log transformation and pareto scaling were applied to the dataset

established OPLS model as shown in Figure S2 and with a variable importance in prediction (VIP) value > 2, along with a narrow jackknife range, reckoner of the VIP value precision confirming the statistical robustness of these analytes.

These four variables were respectively defined as M516a, M516b, M500 and M352 and were characterised by accurate *m/z* measurements and retention times as follows: M516a (*m/z* 516.1699, 6.80 min), M516b (*m/z* 516.1699, 7.00 min), M500 (*m/z* 500.1748, 7.63 min) and M352 (*m/z* 352.1053, 7.85 min). An OPLS model was then developed based on the combination of selected variables to refine the statistical contribution of the four potential biomarkers. The weighted mathematical equation 1 corresponding to the reduced model is presented below; it enables classification of the urine samples based on their status. In this equation, [M516a], [M516b], [M500] and [M352] correspond to the four features signal abundances obtained after processing and transformation (LOESS correction followed by logarithm and Pareto transformations); the weights correspond to the respective OPLS coefficients.

$$Y = 0.520 \times [\mathrm{M516a}] + 0.519 \times [\mathrm{M516b}] + 0.507 \times [\mathrm{M500}] + 0.391 \times [\mathrm{M352}]. \quad (1)$$

3.1.3 | Potential biomarkers annotation

In the present study, the most predictive features highlighted could unfortunately not be identified upon databases search. Nevertheless, these features were further investigated through MS/MS with a mass error lower than 5 ppm. The detected adducts of each potential biomarkers corresponding to the mass 516.1699 (M516a and b), 500.1748 (M500) and 352.1053 (M352) were considered as $[M + H]^+$. This protonated form in ESI^+ is consistent with the results obtained in negative mode. The accurate measured masses in ESI^- were as follows: 514.1601, 498.1649 and 350.0913, and the difference between adduct detected in positive and negative mode corresponds to a difference of two protons. To confirm this hypothesis, lithium cationisation experiments were performed.[43] The formation of *m/z* 522.1818, 506.1865 and 358.1124 ions at the same retention time as M516, M500 and M352 supported the $[M + H]^+$ to $[M + Li]^+$ substitution, which strongly corroborates that the potential biomarkers are protonated species. Then, the possible ionisation in both negative and positive modes suggest the presence of both a basic (e.g., amine group) and/or acidic group (e.g., carboxylic acid group). The odd molecular weight of the four potential biomarkers indicates an odd number of azote.[44] Finally, two neutral losses of 176.0322 observed on M516 (*m/z* 340.1424) and M500 (*m/z* 324.1474) MS/MS spectra suggested two glucuronic esters and thus, an apolar aglycone structure. No glucuronic ester was observed for the M352.

3.2 | Targeted analytical methods implementation on both LC-HRMS and LC-SRM platforms

3.2.1 | LC optimisation and MS fragment ions selection

The feasibility of monitoring highlighted potential biomarkers in a targeted manner using an adapted triple quadrupole instrument was

then studied. Therefore, a transfer of the acquisition method from LC-HRMS towards LC-SRM was initiated. The chromatographic separation has been optimised for high-throughput analysis by a threefold reduction of analytical run time whilst preserving appropriate chromatographic resolution performances as illustrated in Figure 3a. Despite a peak shouldering observed for the M500, the chromatographic separation was considered fitting for the purpose of the study since the potential biomarker M500 corresponded only to one feature after the processing step described in section 2. Furthermore, a LC-PRM fragmentation optimisation for the selected potential biomarkers enabled defining appropriate fragment signals to be subsequently used for LC-SRM targeted acquisition as described below.

3.2.2 | LC-SRM method development and application

SRM parameters were optimised using the QC samples for each of the four potential biomarkers after the evaluation of several MS conditions including source ionisation parameters and collision energy voltages. The defined parameters permitted the detection of the four potential biomarkers of interest as shown in Table 1. Additionally, the chromatographic separation on the LC-SRM chromatography channel was adapted and reduced to 6 min. Thus, a targeted method on the LC-SRM system was successfully transferred as illustrated by chromatograms of the selected SRM transitions in Figure 3b. As depicted, apexes of the transition 516 > 340 was a bit shifted compared to the other transitions and appeared less sensitive as shown in the sample collected before administration. This transition typically corresponds to a glucuronide loss. A large number of metabolites contained in urine are glucuronides and can correspond to this transition explaining a high background noise associated to the shift and the low sensitivity. Despite these observations, transition 516 > 340 was kept in this study because it corresponds to one of the identified fragments of the M516. This transition was not chosen for semi-quantification due to the reasons stated above but may increase detection confidence when combined with the other specific and sensitive transitions selected.

The performance of the model based on LC-SRM acquisition was subsequently assessed and compared to LC-HRMS model by

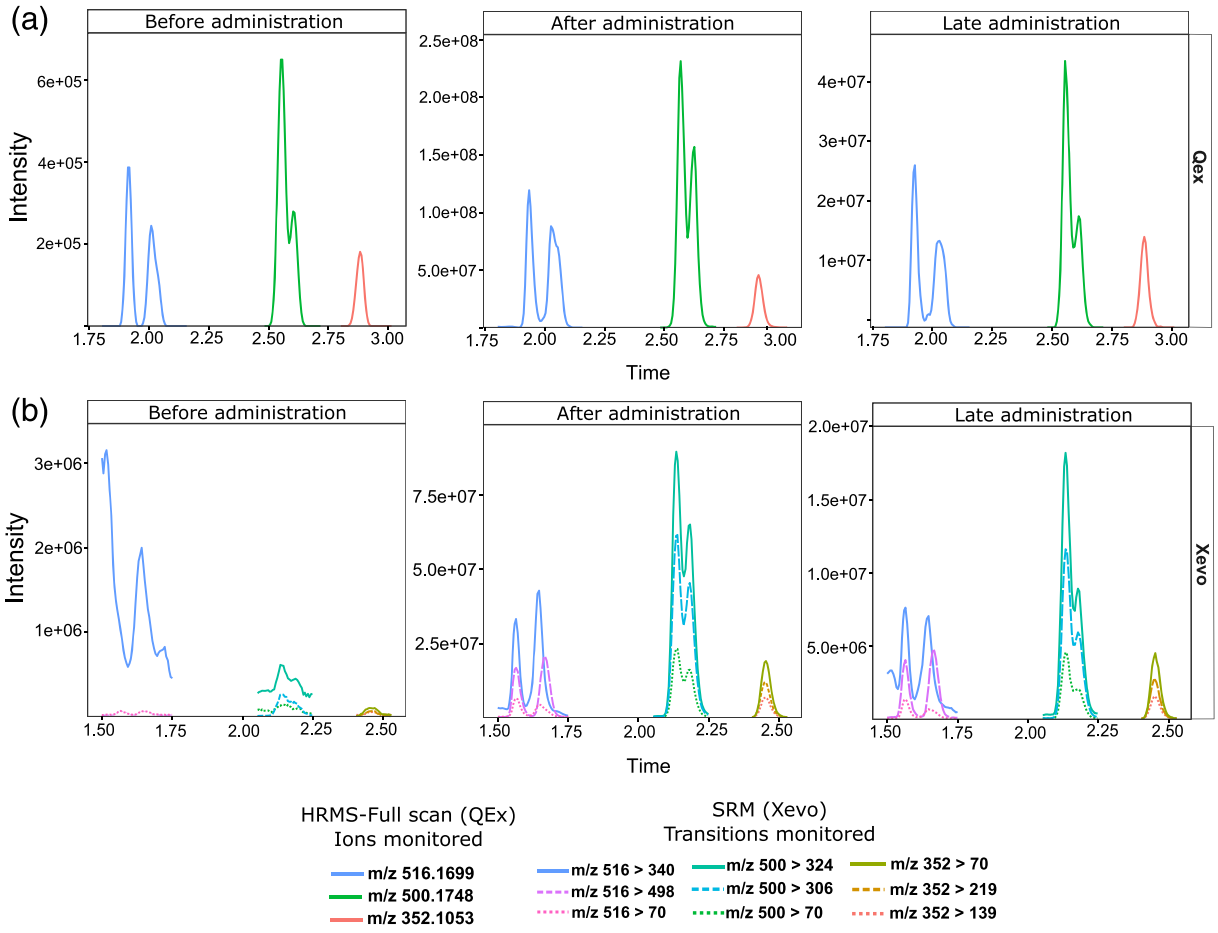


FIGURE 3 Extracted ion chromatograms (XIC) before, after and late administration of testosterone esters cocktail. (a) Accurate masses measured in HRMS-full scan after gradient optimisation UHPLC (Vanquish)-Q-Exactive (Thermo Fisher Scientific). (b) SRM transitions monitored for the four potential biomarkers after gradient optimisation in UPLC (Acquity)-QqQ (Waters)

simultaneously injecting urine samples of the study on both instruments. All potential biomarkers conferred comparable kinetic profiles on both platforms (Figure 4a and b). Four chronological stages could be observed: before administration, early administration, after and late administration. These four stages are in good agreement with the previous observations on the initial OPLS model, which supports the biological relevance and the significance of the selected potential biomarkers (Figure 2).

3.2.3 | Analytical performances evaluation

To evaluate analytical method and transfer performances, a preliminary validation study has been undertaken to assess repeatability (retention time and peak area) and linearity of the response based on the analysis of QC in full scan (LC-HRMS) and LC-SRM. In LC-SRM, the selection of the main transitions of interest for semi-quantification was performed based on their selectivity, sensitivity and linearity of the corresponding responses. The main SRM transitions for M516a, M516b, M500 and M352 were respectively selected as follows: *m/z* 516 > 70, *m/z* 500 > 306 and *m/z* 352 > 70. As shown in Table 2 for both LC-HRMS and LC-SRM workflows, retention times and peak areas repeatability were observed below 3% and 8%, respectively, whilst linearity measurements exhibited a $R^2 \geq 0.99$ for all potential biomarkers (Figure S3). Thus, it could be concluded that the performances of the methods are fit for purpose, regardless of the platform used.

3.3 | Comparison of classification status performances regarding testosterone esters administration

All urine samples collected in the frame of the animal experiment were analysed using the two analytical strategies described above, and their status was predicted on the basis of the model using equation 1. Samples were classified based on the predicted value (*Y*) calculated from the previous equation. The administration of testosterone esters cocktail rapidly induces a significant increase of *Y* value followed by a gradual decrease to a basal state (Figure 5a and b).

Subsequently, different urine samples ($n = 30$) collected from untreated horses, including individual inter-variability (sex, age and practice) presented in Table S1, were analysed using both approaches, and their *Y* values were determined (Figure 5a and b, right panels). The objective was to predict samples status presenting variability and to refine the scope of the prediction model. A Shapiro-Wilk test applied to the obtained values confirmed normal distribution of the control data with a *p value* > 0.5 for both platforms, confirming the suitability of these control samples to establish specific limits to determine the compliant or suspicious status of the urine samples. These

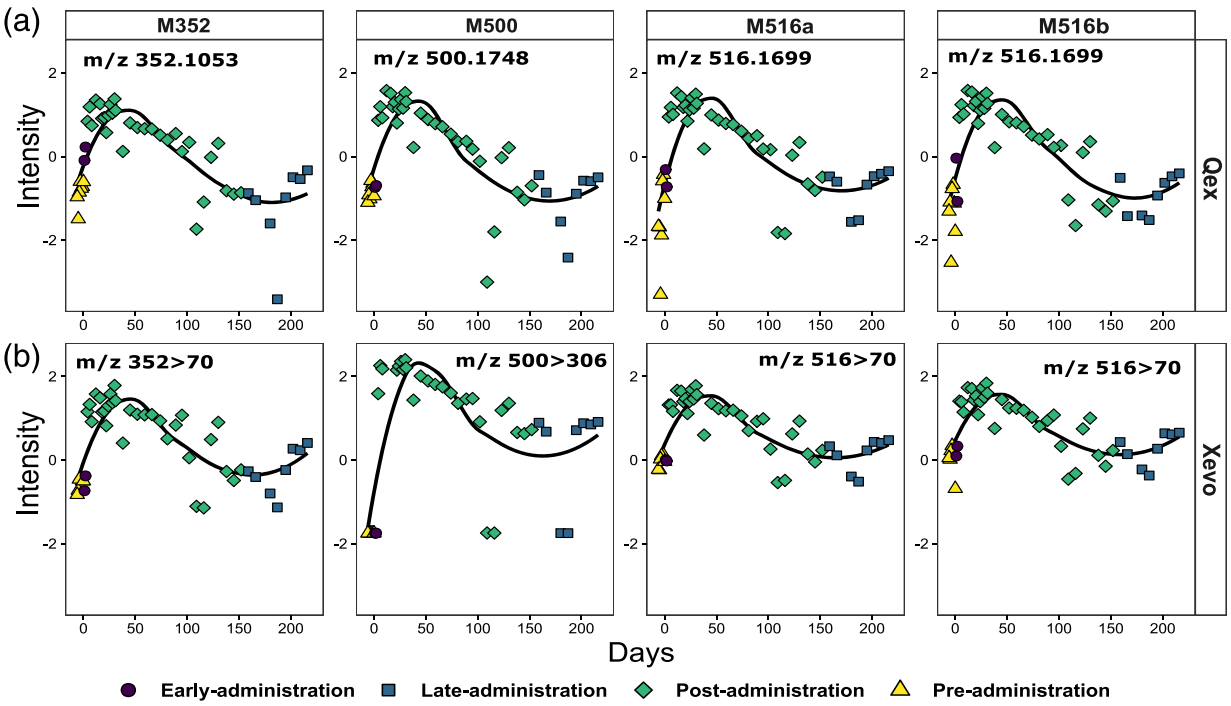


FIGURE 4 Kinetic profiles of the four potential biomarkers contained in *in vivo* urine samples. Results were obtained from the peak area measured after LOESS normalisation, based on QC samples analysed every five injections all along the analytical batch, log transformation and pareto scaling. (a) Monitoring of accurate masses in full scan acquisition in UHPLC (Vanquish)-Q-Exactive (Thermo Fisher Scientific) (QEx) and (b) monitoring of the SRM transition selected for semi-quantification in UPLC (Acquity)-QqQ (Waters) (Xevo) after method transfer

TABLE 2 UHPLC-HRMS and UPLC-SRM method performances

	Putative biomarkers selected			
	M516a	**M516b**	**M500**	**M352**
Ion selected for semi-quantification in full scan	*m/z* 516.1699	*m/z* 516.1699	*m/z* 500.1748	*m/z* 352.1053
Rt repeatability (RSD)	1.93%	2.03%	2.57%	2.88%
Peak area repeatability (RSD)	3.50%	5.00%	3.50%	3.80%
Linearity (R^2)	0.9878	0.9957	0.9992	0.9991
	M516a	**M516b**	**M500**	**M352**
Ion selected for semi-quantification in SRM	*m/z* 516 > 70	*m/z* 516 > 70	*m/z* 500 > 306	*m/z* 352 > 70
Rt repeatability (RSD)	1.60%	1.65%	2.15%	2.50%
Peak area repeatability (RSD)	7.50%	7.15%	4.80%	1.90%
Linearity (R^2)	0.9987	0.9985	0.9995	0.9992

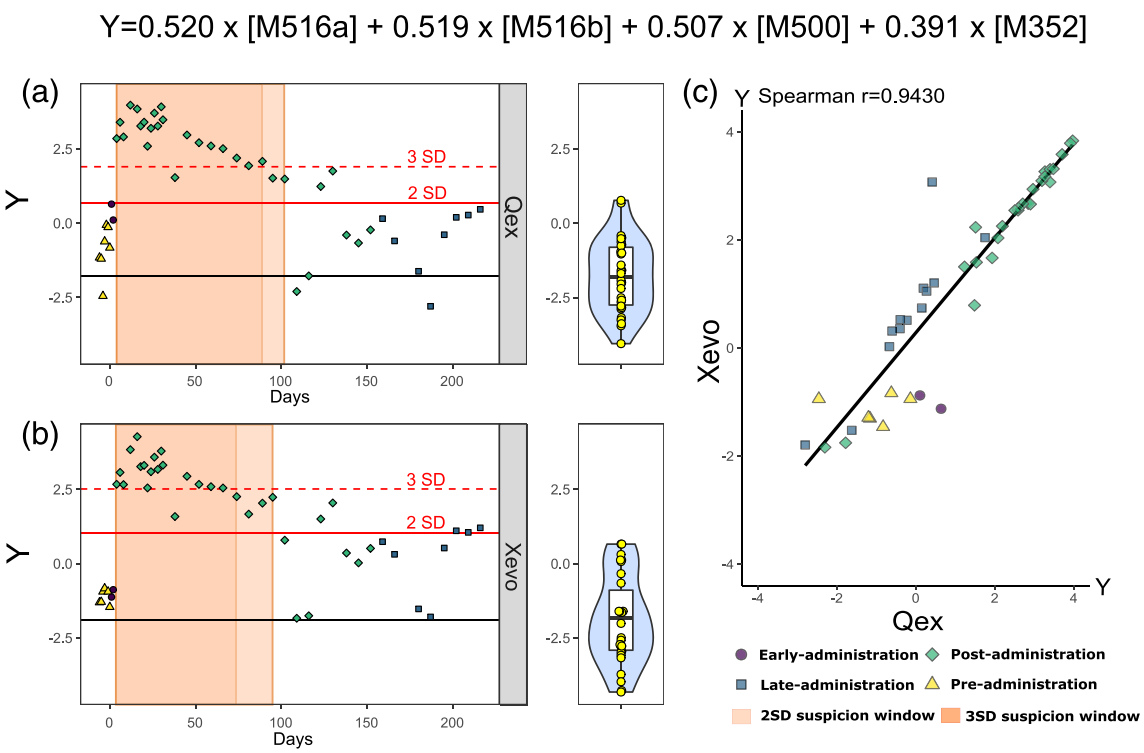


FIGURE 5 Classification of treated and control urine samples based on the equation combined the four potential biomarkers. Results were obtained after peak area measurement, LOESS normalisation, based on QC samples injected every five injections all along the analytical batch, log transformation and pareto scaling. (a) Monitoring of the accurate masses in full scan acquisition in UHPLC (Vanquish)-Q-Exactive (Thermo Fisher Scientific) (QEx) and (b) monitoring of the SRM transitions selected for semi-quantification in UPLC (Acquity)-QqQ (Waters) (Xevo) after method transfer. (c) Spearman correlation between analysis in QEx and in Xevo, *** $p < 0.001$

limits are based on the mean value calculated on the 30 control samples added for instance to 2 or 3 times the standard deviation (SD) which correspond to the 95th or 99th percentile levels of confidence as already described in such context.[22,23] The transfer from HRMS full scan to SRM involves different geometries and parameters of the respective ionisation sources, different acquisition modes and different analysers. Sensitivity depends on these parameters and thus differs from HRMS full scan to SRM analysis.[24] Suspicion limits were then calculated for each instrument (LC-HRMS and LC-SRM). Average control samples *Y* values are indicated as complete black lines (Figure 5a and b left).

In the present study, a 3 SD limit would allow post-administration samples to be classified as suspicious within the 4–89 days post-administration range using LC-HRMS and 4–66 days using LC-SRM approaches (Figure 5a and b), whilst a 2 SD limit enlarges the detection time windows to respectively 4–102 days in LC-HRMS and 4–95 days in LC-SRM. Two control samples approached to the 2 SD cut-off in LC-HRMS analysis (Figure 5a) and were not considered as

suspicious. However, they would require a specific attention, in their respective biological passports, for example. These classification results demonstrate the good performances of the predictive model based on the four potential biomarkers, and the suitability of the implementation of these strategies, regardless of the instrument. Indeed, both MS acquisition modes results (i.e., SRM and HRMS full scan) are highly correlated with a Spearman coefficient > 0.94 (Figure 5c).

Coefficients of the previous equation have also been recalculated from the detection of the four potential biomarkers on LC-SRM, according to the different instruments and acquisition modes. The classification model refinement showed no significant improvement (Figure S4). This observation confirms the applicability of the classification model based on the same equation 1 and constitutes an advantage with the aim to implement such a strategy through different platforms. Thus, the same equation 1 was used in the rest of the study.

In addition, in this context of investigating the impact of steroid administration on the metabolism and considering the influence of gender on their biosynthetic pathway,[45] the effect of sex on the potential biomarkers was evaluated. Comparisons were carried out using the non-parametric Kruskal-Wallis test, and no significant differences between genders (p value > 0.05) were observed (Table S2). To confirm these preliminary results, a more complete study should be performed with a larger number of control samples.

3.4 | Inter-laboratory HRMS method transfer

With the aim to further assess the transferability of such a strategy, the HRMS method (Platform1) was implemented in another laboratory (Platform2) with the same instrument configuration.

Urine samples of *in vivo* study were shipped at −20°C, stored, re-extracted and re-analysed using similar instrumentation and acquisition procedures. The samples were re-classified based on equation 1 using the same limits (2 SD and 3 SD) to compare classification performances. Kinetic profiles of the model values according to equation 1 on both sets of the samples analysed in HRMS-Platform1 and HRMS-Platform2 were similar as shown in Figure 6a and b. Once again, a clear chronological separation (before, early, after and late administration) could be observed. In comparison to the HRMS-Platform1 (Figure 6a) model, the predictive values (Y) determined using HRMS-Platform2 (Figure 6b) were slightly lower due to a possible lower instrumentation sensitivity. A detection window between 4 to 74 days with the suspicion limit set at 3 SD and 4 to 102 days with the 2 SD limit was calculated from the HRMS-Platform2 analysis. These results demonstrated similar classification performances between both platforms.

As illustrated in Figure 6c, the Spearman correlation coefficients between the prediction values measured in the two laboratories (r > 0.85) showed the inter-laboratory reproducibility despite the high variability inherent to the whole process, from storage and shipping to analysis.

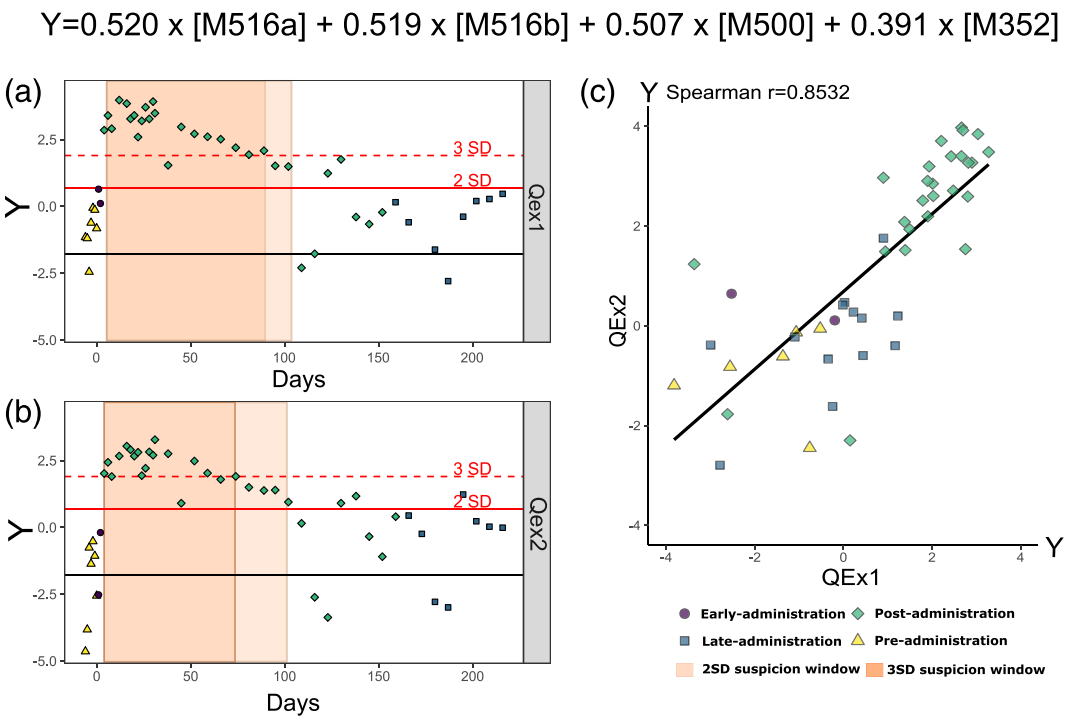


FIGURE 6 Classification of *in vivo* study urine samples based on the equation combined the four potential biomarkers. Results were obtained after peak area measurement, LOESS normalisation, based on QC samples analysed every five injections all along the analytical batch, log transformation and pareto scaling. Monitoring of the accurate masses in full scan acquisition in the HRMS-Platform1 (a) in UHPLC (Vanquish)-Q-Exactive (Thermo Fisher Scientific) (QEx1), (b) in HRMS-Platform2 in UHPLC (Ultimate TM 3000)-Q-Exactive (Thermo Fisher Scientific) (QEx2) and (c) the Spearman correlation between analysis in both laboratories, *** p < 0.001

3.5 | Classification model versus testosterone direct screening

The same urine aliquots from testosterone esters *in vivo* study were analysed with appropriate steroids extraction and LC-PRM analysis. In this context, testosterone, under its sulphated form, was monitored in equine urine as it is the quasi-exclusive form.[46] Selected transitions for the semi quantification were as follows: 367.1585 > 96.9600 for testosterone sulphate and 370.1795 > 97.9660 for testosterone sulphate d3. Based on the suspicion limit of urinary total testosterone set by the IFHA threshold at 20 ng/mL for gelding, suspicion window was estimated for the direct approach as depicted in Figure 7. Direct screening allowed suspicion of testosterone abuse from day 1 to day 66 (Figure 7a), while classification model allows a larger suspicion window starting from 4 days to 89 or 102 days according to 3 SD or 2 SD, permitting a longer detection of suspicious samples than in direct screening approach (Figure 7b).

The large suspicion window (1–66 days) obtained with the direct screening may be explained by the different esters administrated (testosterone propionate, testosterone phenylpropionate, testosterone isocaproate and testosterone decanoate). Minto et al.[47] demonstrated that when diluted in oil prior intra-muscular injection, the variation in ester chains and particularly length and/or aromatic ring structures influence the pharmacokinetics of androgen esters. Thus, early testosterone abuse suspicion may be the result of rapid absorption and metabolisation of the testosterone propionate, whilst effects of other esters are progressive until 66 days. The IFHA threshold of testosterone for gelding refers to free and conjugated forms in urine. As mentioned previously, testosterone is quasi-exclusively excreted in its sulphated form in urine.[46] Therefore, comparison between the classification model and the testosterone direct screening was based using the same suspicion limit value of 20 ng/mL.

In comparison, the classification model showed a similar kinetic with an even larger suspicion window (from 4 to 102 days). This demonstrated the long-term equine physiological alteration following a testosterone esters cocktail administration. Moreover, potential biomarkers responses are delayed by 4 days compared to the direct screening and may correspond to effect delay of the testosterone on the global metabolism. These observations confirm the interest in settling such a model as a complementary strategy to free testosterone direct screening current strategies in early and late post-administration stages.

3.6 | Classification model limitations

3.6.1 | Experimental design

This study relies on an approach involving a single animal treated with a single type of anabolic for more than 200 days to examine temporal changes with metabolic significance. This experimental design although based on a unique animal successfully enabled steroidal markers to be identified and further used in an effective screening method.[23] The results revealed four potential biomarkers combined in

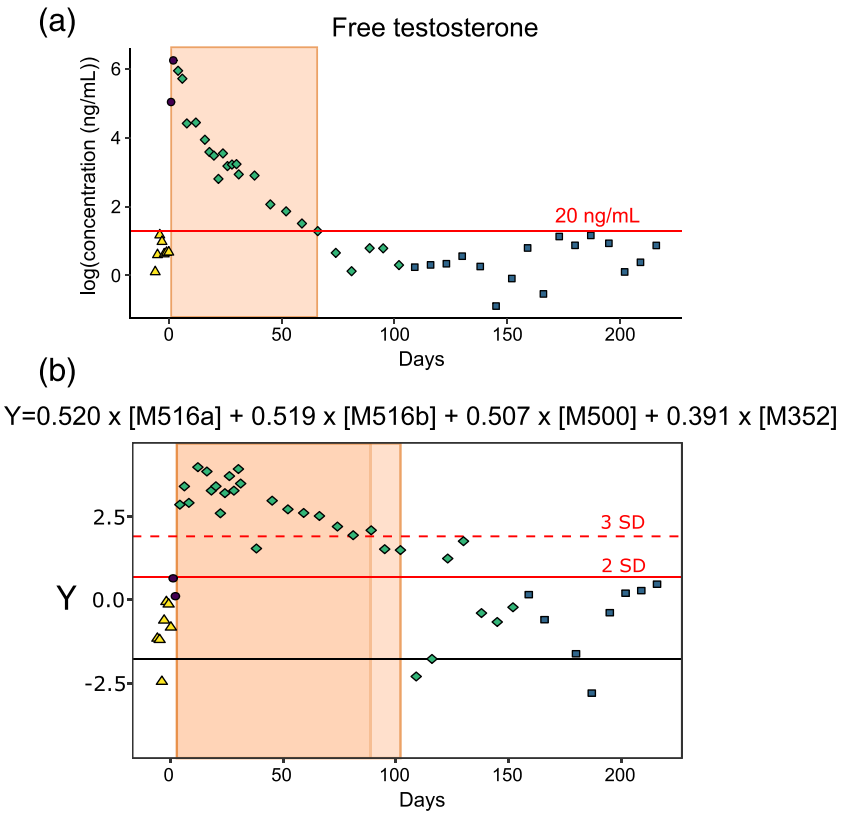


FIGURE 7 Analysis of the *in vivo* study urine samples with a direct screening and the classification model. Comparison between (a) direct approach following free testosterone concentration after log transformation and (b) classification model based on the equation combined the four potential biomarkers. Peak area of the metabolites was obtained after full scan acquisition, LOESS normalisation, based on QC samples analysed every five injections all along the analytical batch, log transformation and pareto scaling. Both approaches were performed in UHPLC (Vanquish)-Q-Exactive (Thermo Fisher Scientific)

a statistical equation signing testosterone esters administration to a gelding up to 102 days post-administration. Generalisation of these results is limited by the lack of biological diversity in this study. Indeed, this experimental design does not allow the assessment of biological response in a context that includes inter-individual variability. This study thus represents only the first brick in a larger project. In order to validate the applicability of the classification model, other testosterone ester treated samples from other horses, including different genders, ages and living conditions, are currently being analysed. This step, known as challenge test, would evaluate the robustness of the potential biomarkers to the variability induced by a larger study cohort.[13,22]

3.6.2 | Identity of the potential biomarkers

In the context of the study, it is important to check whether the biomarkers may be considered as direct metabolite of the administered substance. The glucuronic nature of M516 and M500 may indeed suggest that both compounds were products from phase II metabolism *in vivo* process and could be of exogenous origin or a secondary metabolite of testosterone. Such phase II conjugation is known to induce the inactivation of the compound and to increase its polarity, thus facilitating urinary and biliary excretion of such lipophilic or steroid compounds.[45] A complete structural elucidation is therefore required to conclude on this point. Despite extensive attempts on structural elucidation, this study has not been able to identify the chemical structure of the potential biomarkers yet. Deeper inspection of MS/MS spectra is still in progress. However, the incomplete structural elucidation does not compromise the relevance of the selected potential biomarkers and their potential applications, as already reported.[22]

3.6.3 | Robustness of the classification model

The present study demonstrates that the classification model initially established from HRMS analysis can subsequently be transferred and implemented on another instrument in the same laboratory and on a similar HRMS platform in another laboratory confirming the robustness and reproducibility of the proposed approach. Although the instruments are similar between the two laboratories, the lack of certified reference material to ensure the quality of the analysis and to compare relatively the results obtained from one laboratory to another may be a limitation for future inter-laboratory comparison and validation of the method.

The performances of the classification model with regard to different anabolic treatments, different molecule classes and different horses including different physiological and environmental aspects remain to be studied.[22] Due to the lack of treated samples from other horses and other treatment, the scope and the performances of the classification model on treated samples was not defined in the current study. Further analysis will also be made such as population studies, additional *in vivo* studies or confounding factors assessment, to validate the potential biomarkers as a new tool for doping control.

3.6.4 | Toward implementation of the classification tool

Once the validation process described above is finalised and the method meets the screening requirements, it could become a candidate for routine implementation in analyses carried out for control purposes. In case no reference standard is available, it will consequently involve the resort to a panel of precautions to be taken, such as the preparation of QC samples, the use of a set of pseudo-ISs and the characterisation of both compliant and non-compliant reference samples all along the analytical sequence.

4 | CONCLUSION

In this study, a successful inter-device (LC-HRMS to LC-SRM) and inter-laboratory (HRMS-Platform1 to HRMS-Platform2) transfer of a metabolomics classification model is presented. The developed tool displays improved performances in term of suspicion window compared to a direct approach. The results obtained are encouraging, and the measurements of the four potential biomarkers upon different instruments and laboratory transfers highlight that the markers can be considered as reproducible signals. Moreover, independent to the selected system (HRMS or SRM), the simple sample preparation and rapid transfer from non-targeted to targeted analysis constitute a major advantage for possible routine application.

This proof-of-concept study will be further completed with specific research focussed on complete structural elucidation. In addition, robustness and performances of the classification model will be assessed using additional cohort of the study (population studies, additional *in vivo* studies, confounding factors assessment, etc.) to validate the potential biomarkers as a new tool for doping control.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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